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REVIEW

Engineering Exosomes for Therapeutic Applications: Decoding Biogenesis, Content Modification, and Cargo Loading Strategies

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Abstract: Exosomes emerge from endosomal invagination and range in size from 30 to 200 nm. Exosomes contain diverse proteins, lipids, and nucleic acids, which can indicate the state of various physiological and pathological processes. Studies have revealed the remarkable clinical potential of exosomes in diagnosing and prognosing multiple diseases, including cancer, cardiovascular disorders, and neurodegenerative conditions. Exosomes also have the potential to be engineered and deliver their cargo to a specific target. However, further advancements are imperative to optimize exosomes' diagnostic and therapeutic capabilities for practical implementation in clinical settings. This review highlights exosomes' diagnostic and therapeutic applications, emphasizing their engineering through simple incubation, biological, and click chemistry techniques. Additionally, the loading of therapeutic agents onto exosomes, utilizing passive and active strategies, and exploring hybrid and artificial exosomes are discussed.

Keywords: exosome, engineered exosomes, drug delivery, diagnostics, therapeutics

Introduction

Exosomes have gained increasing interest in therapeutic and diagnostic applications. Studies have demonstrated the protection and recovery potential of exosomes in several diseases. For example, the anti-inflammatory and neurogenesis effects of stem cell-derived exosomes in mouse models of traumatic brain injury, stroke, Alzheimer's disease, and Parkinson's disease have shown the potential of exosomes as therapeutic nanovesicles (Table 1). The contents of exosomes can affect the host cells since they transport different nucleic acids (DNA, various RNA, miRNA, and siRNA), lipids, and proteins. In addition to using intact exosomes, researchers are attempting to modify them for precise targeting. Exosomes may transport siRNA, miRNA, and chemotherapeutic drugs such as doxorubicin and gene editing systems.[1] Wood's group delivered siRNA to the mouse's brain through the systemic infusion of exosome-derived dendritic cells, significantly decreasing the BACE1 mRNA and protein.[2]

Researchers have made extensive efforts to bring exosomes into clinical settings. Meanwhile, artificial exosome engineering is rapidly advancing, faithfully replicating the functionality of natural exosomes. In this field, modifications can be made to either the surface[12–18] or cargo of the exosome.[19–24] This engineering can involve modifying the parental cell to produce exosomes tailored for specific cargo loading[20–22,24,25] or surface modification,[17] or it can be applied directly to the exosome.[24–26] Another method for creating artificial exosomes is mixing synthetic liposomes with natural exosomes to produce hybrid exosomes.[1,27,28]

Numerous exosome-related patents exist, such as WO2015110957A3, in which Joel de Beer et al patented a method using electrostatic interactions to guide targeted interactions between exosomes and liposomes. In this patent, incorporating the pH-sensitive lipids in liposomes prevents the hybridization of the exosome-exosome or liposome-liposome.[29] Marban et al (US11660355B2) demonstrated that exosomes from various cell sources can be engineered with reporters,

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Table 1 Using Stem-Cell Derived Exosomes or Engineered Exosomes to Target Neurodegenerative Diseases and Cancers

Disease	Type	Origin of Exosomes	Cargo	Model	Target	Administration	Ref.
Neurodegenerative disease	Alzheimer's	MSC	siRNA	AD mice model	Strong knockdown BACE1	Systemic injection	[2]
	Alzheimer's	MSC	Contents of the intact Exosomes	AD mice model	B amyloid	Subventricular zone microinjection	[3]
	Parkinson's	MSC	Curcumin	PD model mice	Mitigation of α- synuclein aggregates	Intranasal	[4]
	Parkinson's	Raw 264.7	Catalase	PD model mice	Anti-inflammatory effect	Intranasal	[5]
	Autism	Human Umbilical mesenchyme stem cell	Contents of the intact Exosomes	Autistic mouse	Anti-inflammatory effect and behavior improvement	Intranasal	[6]
	Stroke	Human neural stem cell	Contents of the intact Exosomes	Murine thromboembolic stroke model	Neural injury	Tail vein injection	[7]
	Multiple sclerosis	Human MSC is stimulated by INFγ	Contents of the intact Exosomes	Experimental autoimmune encephalomyelitis (EAE) mouse model	Alleviation of neuro-inflammatory and demyelination	Intravenous	[8]
	Traumatic brain injury (TBI)	Human adipose mesenchymal stem cell	Contents of the intact Exosomes	TBI rat	Neurogenesis	Intracerebroventricular microinjection	[9]
	Huntington's	U87 glioblastoma cells	Hydrophobicity modified siRNA	Wild-type FVBNj mice	mHtt mRNA	Unilateral infusion (Cerebellar microinjection)	[10]
Cancers	Oral Squamous cell carcinoma	Normal fibroblasts transfected with Epstein–Barr Virus Induced-3 (EBI3) cDNA was electroporated with siRNA of lymphocyte cytoplasmic protein 1 (LCP1)	siRNA against lymphocyte cytoplasmic protein 1	SAS and HSC-3 tumor-bearing mice (Nude mice by engrafting the tumor bearing cell lines)	Lymphocyte cytoplasmic protein 1 mRNA	Intravenous	[11]
	SKBR3 cell line (HER2+)	Transfected MSC with LAMP2b-DARPin gene	Dox	In vitro culture	HER2 receptor, SKBR3 cell line (HER2+)	In vitro culture	[12]

Notes: The RAW 264.7 cell line is a commonly used mouse macrophage cell line in biomedical research. The abbreviation RAW stands for "Rat Ascites Tumor", and 264.7 refers to the clone number of the cell line. SAS and HSC-3 are oral squamous cell carcinoma-derived cell lines. SKBR3, a human breast cancer cell line isolated by Memorial Sloan–Kettering Cancer Center in 1970, is widely used for studying breast cancer and is characterized as HER2-positive (HER2+), indicating overexpression of HER2.
Abbreviations: MSC, Mesenchymal Stem Cells; siRNA, small interfering RNA; AD, Alzheimer's Disease; BACE1, beta-site amyloid precursor protein cleaving enzyme 1; PD, Parkinson's Disease; INFγ, Interferon-gamma; mHtt, mutation of the gene huntingtin (htt); HER, human epidermal growth factor receptor 2; DARPin, designed ankyrin repeat protein; Dox, Doxorubicin hydrochloride.

aptamers, or different segments of antibodies for effective tissue targeting. They modified the exosomes with a pegylated phospholipid linked to streptavidin, allowing biotinylated molecules to be added to the surface for improved tissue targeting and signal tracking.[30]

Noyes et al in Codiak BioSciences company (WO2020191369A1) demonstrated a significant advancement in extracellular vesicle (EV) purification with a large-scale process involving multiple chromatography steps using cation and anion exchange resin chromatography. This method yields highly purified EVs, characterized by reduced protein impurities such as perlecan, agrin, and/or total proteins, resulting in enhanced potency, uniformity, or a combination of both.[31]

In another patent (US20240075100A1), Choi et al introduced an effective technique for loading proteins into exosomes using an innovative optical reversible protein reaction. Their patent is based on the natural photoreaction of the photoreceptor cryptochrome 2 (CRY2) and CRY-interacting basic-helix-loop-helix 1 (CIB1) protein module, originally found in Arabidopsis thaliana under blue light. By altering CD9 with a shortened version of CIB1 and engineering the protein cargo to contain CRY2, they enabled the interaction to be triggered by blue light, facilitating cargo loading inside the exosome. Switching off the light tended to release the protein cargo.[32]

In less than a decade, pharmaceutical companies have increasingly invested in exosome research as nucleic acid carriers, potentially replacing the almost four-decade-old viral gene therapy approach.[33] In this paper, we provide a comprehensive review of the biomedical applications of exosomes in diagnostics and therapeutics. We also explore different methods for engineering exosomes to enhance cargo loading and targeting capabilities.

Exosome Biogenesis

The process of exosome release from a cell involves three key steps: (i) the generation of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs), (ii) the translocation of MVBs to the plasma membrane, and (iii) the fusion of MVBs with the plasma membrane. In the fusion step, cholesterol levels may impact the release of exosomes, as only high-cholesterol MVBs release exosomes (Figure 1). Factors other than cholesterol affecting exosome release include

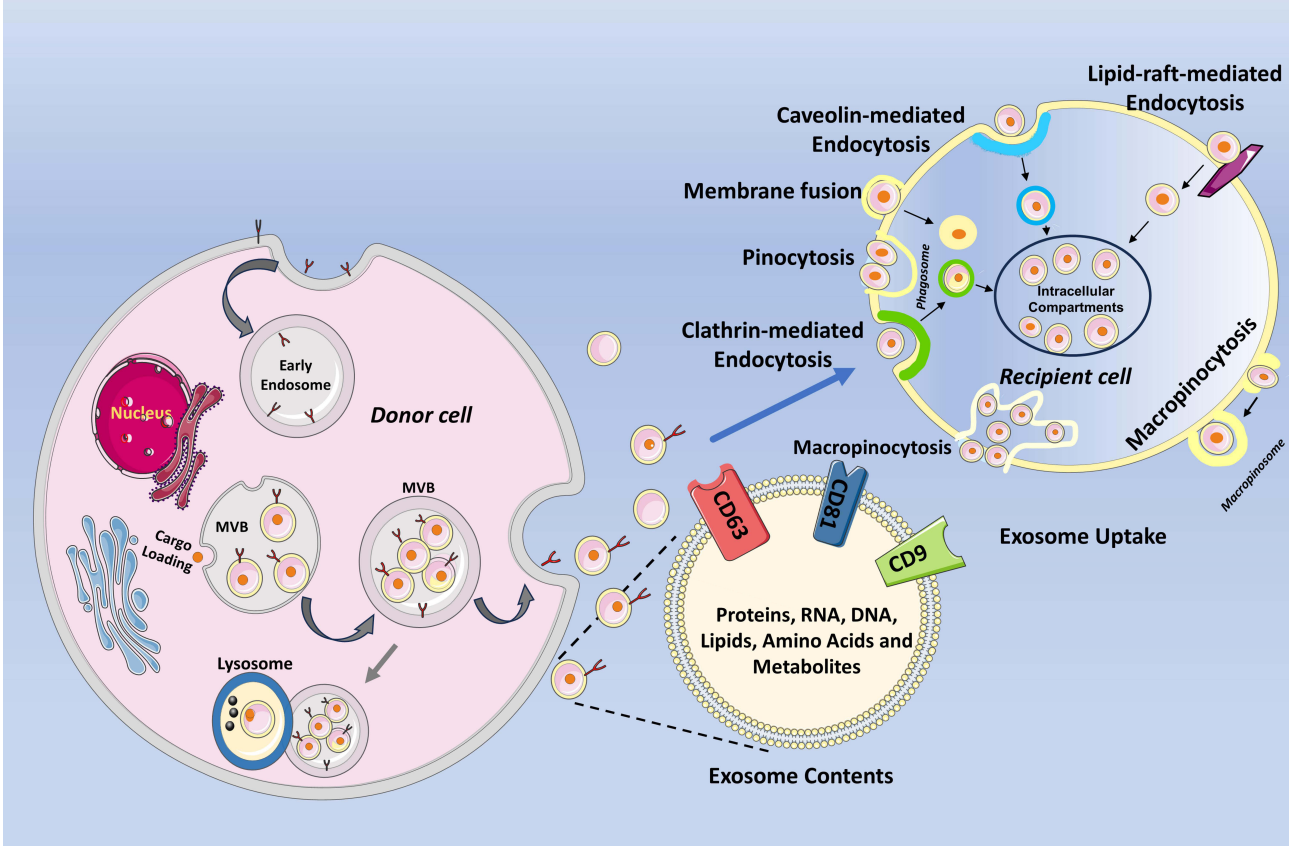

Figure 1 The biogenesis, contents, and cell uptake methods of the exosome. Exosomes, identified by markers CD63, CD9, and CD81, form when multivesicular bodies (MVBs) fuse with the plasma membrane, encapsulating nucleic acids, proteins, lipids, metabolites, and small molecules. MVBs can either be digested by lysosomes or release exosomes. Once released by donor cells, exosomes are absorbed by recipient cells through various pathways: pinocytosis, clathrin- and caveolin-mediated endocytosis, lipid raft endocytosis, macropinocytosis, or membrane fusion. Within recipient cells, exosome contents are sorted into various compartments or induce specific cellular responses. The figure was drawn using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution (https://creativecommons.org/licenses/by/4.0/).

proteins, lipids, and cell types. The endosomal sorting complex required for transport (ESCRT) machinery is vital for exosome biogenesis and cargo loading. ESCRT comprises protein complexes of ESCRT (0, I, II, III) and the AAA ATPase Vps4 complex (AAA stands for ATPases associated with various cellular activities).[34] Exosome secretion may be affected when levels of these proteins are reduced or depleted. For example, depletion of Hrs and Tsg101 (tumor susceptibility gene 101) of ESCRT-0 and STAM1 of ESCRT-1 reduces exosome secretion; nevertheless, knockdown of VPS4B (related protein) enhances exosome secretion. The depletion of ALIX, another related protein, reduces exosome secretion and alters its protein loading.[34] ALIX is also involved in the transferrin receptor sorting and loading of miRNAs in rat and human liver cells, respectively.[35]

The ESCRT-independent pathways are also crucial in exosome sorting and loading.[35] The ESCRT-independent machinery includes tetraspanins, sphingomyelin, and ceramide,[36] as well as lipid rafts.[37] The tetraspanins (eg, CD9, CD63, CD81) have four transmembrane domains essential for exosome production and budding. The C-terminal of the CD9 interacts with the cytoskeletal protein (actin) and results in exosome fission from the cell.[36] CD63 is involved in exosome packing and releasing since its knockdown decreases exosome release.[38] In certain cell lines like Oli-neu cells (oligodendroglial precursor cell line), ceramide has a role in exosome release and promotes membrane budding. However, in bone metastasis cells (PC-3 cells), blocking neutral sphingomyelinase 2 (which produces ceramide from sphingomyelin) did not affect exosome release.[34] Ceramides, a type of sphingomyelin, have a cone shape and help with blebbing and multivesicular evagination in the inner leaflet of the plasma membrane. The significance of ceramides in exosome secretion is supported by D609, an inhibitor of sphingomyelin converter that upregulates ceramide by inhibiting its conversion to sphingomyelin.[39]

Methyl-β-cyclodextrin, a lipid raft inhibitor, shows the role of lipid rafts in the exosome sorting process. Lipid rafts, specialized membrane domains enriched in cholesterol, sphingolipids, and glycosylphosphatidylinositol (GPI)-anchored proteins are pivotal in lipid raft-mediated cargo loading into exosomes. Proteins associated with these lipid rafts are effectively sorted into ILVs within MVBs. Shifting membrane proteins from non-lipid raft domains to lipid rafts could potentially enhance their incorporation into ILVs within MVBs. Upon fusion of MVBs with the plasma membrane, exosomes are released into the extracellular space, carrying the sorted cargo molecules. However, disruption of lipid rafts using methyl-β-cyclodextrin impedes the secretion of these proteins through exosomes. In summary, the involvement of lipid rafts in the exosome sorting process is demonstrated by their crucial role in facilitating cargo loading into ILVs, ultimately shaping the composition and function of exosomes.[37]

Exosome Contents

Exosomes contain a diverse range of biomolecules, which include lipids, proteins, nucleic acids, and other small molecules (Figure 1). The precise content of exosomes can vary depending on the cell type of origin and the physiological state of the parent cell. In the next section, we have outlined the diverse contents of exosomes. We have divided these into three main categories: lipids, proteins, and nucleic acids.

Lipids

Exosomes are composed of and transport different lipids, such as cholesterol, fatty acids, and eicosanoids, along with their related metabolic enzymes. Exosomal compounds may influence lipid transporters, nuclear transcription factors, programmed cell death, inflammatory and immunological responses, and other lipid metabolism-related activities.[40] The imbalance in lipid metabolism causes atherosclerosis, cancer, non-alcoholic fatty liver disease, obesity, and Alzheimer's disease.[41] Following ceramide accumulation in neural cells, astrocyte-derived exosomes may promote brain cell death and Alzheimer's disease. The exosomal miRNA regulates the lipid-metabolism genes like peroxisome proliferators-activated receptor-γ, -α (PPAR-γ and -α). Exosomal miR-155 and miR-27 inhibit the expression of PPAR-γ. Meanwhile, PPAR-γ controls the intake, storage, and metabolism of lipids in the liver.[41] Inactivation of PPAR-α by exosomal miRNAs has been proposed to lead to obesity, where systemic injection of the obese mice's exosome drives lean animals to acquire obesity-like characteristics. Mice with adipose-specific RNA interference develop hypertriglyceridemia, glucose intolerance, and dyslipidemia.[42] Lipid metabolism also influences the biological processes of exosomes, such as biosynthesis and interactions with recipient cells. For example, researchers discovered that cholesterol efflux by

different transporters affects exosome trafficking. ABCA1 (ATP-binding cassette transporter A1) increases exosome release, whereas SR-B1 (scavenger receptor class B member 1) may restrict exosome absorption in recipient cells.[41]

Proteins

Exosomal proteins are suitable biomarkers for the diagnosis of various diseases.[43] Compared to other components, they are stable, and detecting a small amount of exosomal surface proteins is possible. Exosome tumor markers include epidermal growth factor (EGFR),[44] epithelial cell adhesion molecule (EpCAM),[45] EphA2 (ephrin type-A receptor 2),[46] E cadherin (epithelial cadherin), and PD-1 (Program-death-1).[47] Exosome proteins affect cancer angiogenesis, growth and metastasis, and therapeutic resistance. The protein profiles of exosomes can potentially be used as a noninvasive source of biomarkers for cancer diagnosis and therapy.[43] However, exosomes can serve as a platform for generating aggregated abnormal proteins like α-synuclein and tau in Parkinson's and Alzheimer's, respectively. As reviewed by Howitt and Hill, studies revealed that these proteins can be disseminated to normal brain cells, contributing to the onset and progression of neurodegenerative disorders.[48]

Enhancing our understanding of protein trafficking within exosomes holds potential for refining diagnostic and therapeutic strategies. Notably, proteomic analyses have demonstrated shifts in the protein composition of exosomes derived from cancer cells. An illustrative case is evident in glioblastoma cells, where proteomics revealed the presence of oncogenic modifications such as the constitutively active expression of EGFR vIII (epidermal growth factor receptor variant III). This phenomenon leads to an exosomal cargo enriched with numerous pro-invasive molecules, underlining the dynamic nature of exosome-mediated protein transport.[49]

Researchers have demonstrated that Lamp2A plays a pivotal role in sorting proteins into exosomes. Depletion of this protein led to a substantial reduction in exosomal protein content, all of which shared a specific amino acid motif—KFERQ. This finding aligns with a mechanism wherein proteins containing a pentapeptide sequence, which exhibits biochemical similarity to the KFERQ motif, are selectively loaded into a distinct subpopulation of exosomes, contingent upon the presence of the membrane protein LAMP2A.[50]

Nucleic Acids

Exosomes can contain a variety of nucleic acids, including DNA and RNA. Exosomes can carry viral,[51] bacterial,[52] and parasite DNA and RNA during infections.[53] For example, the DNA of *Plasmodium falciparum*, the parasite responsible for causing malaria, has been detected in the exosomes of infected red blood cells.[54] This DNA facilitates the spread of the parasite to other red blood cells, contributing to the progression of the disease.[55] Furthermore, Jeppesen et al showed that exosomes lack double-stranded DNA and incorporated histones.[56] So, exosomes cannot transport genomic DNA from the cell's nucleus, which has crucial implications for understanding exosome activities in many biological processes, such as disease progression and immunological response.[56]

The RNA content of exosomes comprises various RNAs: rRNA, circular RNA, miRNA, and long non-coding RNA. The miRNA can affect the gene expression of recipient cells, and they can be sorted. The target cells can affect the type of miRNA in the exosome since the knockout of the specific gene in target cells can decrease the miRNA sorting and packaging in the donor cells. Researchers showed that the miRNAs inside the exosome have specific motifs called EXO-motifs. This motif and its associated proteins regulate the loading of miRNA. RNA binding proteins, including Y-box protein 1, annexin A2, hnRNPA2B1, and SYNCRIP, are implicated in the loading of specific miRNAs—miR-144 and miR-233, viral RNA, miR-198, and Exo-motif RNA of GGCU, respectively.[37] In addition, a short motif regulates the loading of microRNAs into exosomes, and this short motif is recognized by hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2B1).[57] Manka et al designed aptamers with lipid raft and exosome binding motifs, termed RAFT and Exo motifs. The researchers meticulously assessed these aptamers' binding affinities within a liposome-based exosomal model, shedding light on their interactions. They assessed their affinity using a liposome exosomal model. MiRNAs containing RAFT (UUGU, UCCC, CUCC, CCCU) and Exo (GGAG, UGAG, CCCU, UCCU) motifs were encapsulated, suggesting potential for targeted therapeutic RNA transport. These motifs also imply specific lipid raft interactions and functional implications.

Exosome Uptake

Internalization of exosomes may occur via several processes, including membrane fusion, lipid-raft mediated endocytosis, phagocytosis, macropinocytosis, caveolin- mediated and clathrin-mediated endocytosis (Figure 1). Interactions between cells and exosomal proteins and glycoproteins may affect internalization. The uptake of exosomes by recipient cells can influence the function of the cell under both physiological and pathological conditions. Understanding the mechanisms behind this process can help study the various potential applications of this vesicle in cell functioning tests and theragnostic platforms, both diagnostic and therapeutic fields.[58]

Biomedical Applications of Exosomes

Subsequent investigations into exosomes hold the key to unveiling their substantial potential in translational medicine. This endeavor promises to usher in innovative pathways for advancing robust clinical diagnostics and sets the stage for developing highly effective therapeutic strategies. Coined as "exosome theragnostic", the utilization of exosomes in these dual capacities underscores their pivotal role in bridging the gap between diagnostics and therapeutics and shapes the future landscape of medical research and application.

Diagnostics

Exosome profiling monitors donor exosomes to prevent fatal organ damage and compensable diagnosis.[59] For example, Hu et al clinically detected antibody-mediated heart rejection. The donor exosome's human leukocyte antibodies identify antibody-mediated rejection.[60] Furthermore, exosome biomarkers can potentially detect and monitor pregnancy complications.[59] Salomon et al discovered an increase of miR-486-1-5p and miR-486-2-5p in preeclampsia, which is helpful for early detection.[61] The miRNAs of the exosome have a critical role in the theragnostic field, both diagnostic biomarkers and treatment strategy.[59] For instance, overexpression of miR-9 and miR-124 are the biomarkers of acute ischemic stroke detected in serum exosomes.[62] Exosomal DNA with more biological information than circulating DNA is another source of accurate prognosis prediction.[59]

Neurodegenerative Disorders and Exosome

Detecting neurodegenerative diseases at an early stage can help mitigate their most severe consequences. Hence, finding promising peripheral indicators is crucial. Aside from the results of NMR and mass spectrometric biometabolite research, exosomes may be a feasible option for identifying and treating neurodegenerative disorders.[63] Some exosome biomarkers of neurodegenerative diseases include the formation of amyloid-beta, amyloid precursor proteins,[64] and lactoferrin[65] in Alzheimer's diseases, aggregation of misfolded alpha-synuclein,[66] muted protein deglycase (DJ-1)[67] in Parkinson's disease, lower activity of acetylcholinesterase[68] and phosphorylated tau[69] in Parkinson diseases, superoxide dismutase 1 (SOD1)[70] and tar-DNA binding protein-43 (TDP-43) in Amyotrophic lateral sclerosis[71] and mutant huntingtin protein (mHtt) in Huntington disease,[72] as well as miRNA changing in all diseases.[73] In Alzheimer's disease, released amyloid-beta binds glycoprotein and glycolipid on the exosome. Lim et al demonstrated a distinct preference for aggregated amyloid-beta (Aβ42) to bind with exosomes. This finding suggests that exosomes are a stronger predictor of disease progression than free amyloid-beta. They innovated a sensitive plasmon nanostructure to detect Aβ42 and CD63 simultaneously in the limited number of 200 exosomes in Alzheimer's plasma samples.[74] The various RNAs in the exosome also have the potential application in monitoring neurodegenerative diseases. Sue et al settled a ceRNA network of mRNA, miRNA, lncRNA, and circRNA, which shows Alzheimer's progress. They indicated the rising and falling of miR-27a-5p and miR-148a-5p in the exosome of the Alzheimer's mice model, respectively.[75]

Cancer Diagnosis and Exosomes

Exosomes are also important in cancer diagnosis. Exosome-mediated interactions between tumor, immunological, and stromal cells in the tumor microenvironment may aid cancer diagnosis. Exosome contents and their signaling abilities impact cancer development by transferring factors that influence growth, transformation, and survival, facilitating communication among cancer cells. Screening for exosomal proteins is a standard method for detecting cancer

biomarkers in various malignancies. For instance, caveolin and CD63 of blood exosomes rise in oral cancer, and exosomal CD81 and prostate antigen increase in prostate cancer.[76] A particularly significant cancer marker, Glypican 1+ (GPC1+), is prominently present on exosomes released by cancer cells. Despite high costs and potential for false positives in magnetic resonance imaging (MRI), detecting circulating GPC1+ exosomes with mutated KRAS has proven effective in identifying early-stage pancreatic cancer in both mice and humans. This finding underscores the potency of exosomal analysis in achieving accurate cancer diagnosis.[77]

Moreover, the screening of exosome DNA in pancreatic cancer has shown a higher detection rate of KRAS mutation in the early stages, and it may be a supplemental diagnostic technique.[78] Non-coding RNA may be another cancer marker, as the exosomal miRNA-21 and lncRNA-ATB significantly increase in hepatic cancer (hepatocellular carcinoma).[79] Malignant forms of ovarian, esophageal, colorectal, liver, and breast cancers express miRNA-21 in blood circulation exosomes.[76] In addition, the low miRNA let7 and the large quantity of RNA-binding protein Lin28B released by breast cancer stem cells may cause immunosuppression and lung metastasis.[80] According to Yang et al circPSMA1, a circular non-coding RNA in the exosome may be a marker of triple-negative breast cancer (TNBC); circPSMA absorbs and suppresses miR-637, followed by metastasis and immunosuppression.[81] Nevertheless, given the limited expression of miRNA, enhancing the sensitivity of detection methods at these minute quantities is crucial to ensure the smooth incorporation of these discoveries into clinical practices.[76]

Additionally, encapsulating carbon dots inside exosomes represents a promising approach for imaging tumors with high sensitivity, specificity, and biocompatibility. Recent research showed macrophage exosomes coated boron carbon nanodots after a two-hour coincubation at 37°C. The encapsulated boron carbon nanodots in the mouse model demonstrated a remarkable ability to accumulate in glioblastoma tumors and cross the blood-brain barrier. However, it is worth noting that this research also detected nanoparticle signals in other organs, such as the kidney, lung, and liver.[82] In this study, the carbon dots lacked specific modifications on the surface for entrapment inside the exosomes. They could be released due to gradient differences following loading and subsequent washing steps. Furthermore, measuring loading and encapsulation efficiency percentages using UV-vis did not guarantee that the signal was confined within the exosome, as it could also be present in the solution due to potential release from the exosome-based on gradient differences.

Exosomes are crucial in transporting disease-specific biomolecules, enabling noninvasive sampling for early diagnostics. Beyond their diagnostic applications, the therapeutic potential of exosomes will be discussed in the following section.

Therapeutic Applications

Studies have investigated exosomes as a drug delivery system for treating neurodegenerative and cardiovascular diseases and cancer. According to Pinnell and Tieu's review, studies have indicated that assessing α-synuclein levels in both plasma and saliva is a potential marker for Parkinson's disease diagnosis.[83] Targeting α-synuclein mRNA by exosome-loaded antisense oligonucleotide enhanced the movement in the Parkinson's mice model (α-synuclein A53T).[84] Furthermore, catalase-loaded exosomes demonstrated remarkable brain delivery through nasal administration in Parkinson's mouse models.[5] Kojima et al transferred the catalase mRNA in the brain of Parkinson's mice model by subcutaneous implantation of engineered cell lines, which frequently released exosomes; these exosomes include the small reporter at 3′ terminal of CD63, RVG-Lamp2, and catalase mRNA for exosome tracing, brain targeting and anti-inflammatory effects respectively. This study addressed the challenge of rapid exosome removal through intravenous injection.[85] In Parkinson's model mice, dopamine-loaded blood cell-derived exosomes showed more than 15 times greater brain distribution, effectiveness, and less toxicity than free dopamine.[86] In neurological applications, intranasal delivery of this nanocarrier is more effective than intravenous, which is cleared in the kidney and liver.[6] Exosomes can be the carriers of siRNA to control gene expression. The siRNA delivery and gene silencing to the brain of the Huntington mouse model were significantly improved through engineered exosomes. Dar et al ameliorated siRNA exosome loading by creating an RNA binding system. They incorporated GAPDH (glyceraldehyde-3-phosphate dehydrogenase) with the phosphatidylserine binding domain and the RNA binding protein for attaching to the exosome surface and siRNA binding, respectively. The siRNA loading onto the exosome's surface indicated a significant decrease in the activity of

the Huntington's gene following injection into mice. This study demonstrated that attaching siRNA to GAPDH protected it from degradation by nucleases in serum.[18]

Exosomes have several potential benefits over current cancer immunotherapy strategies, including carrying various antigens, activating various immunological pathways, and eliciting long-lasting immune responses. They can deliver tumor-associated antigens (TAAs) to antigen-presenting cells (APCs) like dendritic cells (DCs), which may activate T cells and generate an immune response against cancer cells. Exosomes are isolated from the patient's tumor cells and then incubated with DCs in vitro. This incubation allows the loading of tumor exosomes onto the DCs, which may result in the presence of TAAs on the DC surface. T lymphocytes are then stimulated to detect and react to TAAs due to this presentation. Besse et al showed the boosting effect of this immunotherapy on the natural killer cells in patients with advanced non-small cell lung cancer (NSCLC). Exosomes are a viable treatment option for cancer patients since they are well-tolerated and have little toxicity.[87] Using modified exosomes to transport drugs shows promising results in controlling cancer tumor development. Suppressing CD47 and its linking to SIRP-α on the mast cells helps cancer immunotherapy. Anti-CD47 and anti-SIRP designed exosomes were activated in mice's acidic tumor microenvironment and reduced tumor growth.[88]

Exosomes have recently attracted interest as a possible transporter for therapeutic compounds, particularly those in gene editing. Researchers have harnessed these carriers to transport CRISPR Cas9 in various forms, including DNA, RNA, and ribonucleoprotein complexes (RNPs). Kim et al utilized cancer cell-derived exosomes to deliver CRISPR Cas9 plasmids, effectively inhibiting poly ADP-ribose polymerase-1 and inducing apoptosis in ovarian cancer.[89] In another study, exosomes from red blood cells served as carriers for the mRNA form of CRISPR Cas9, demonstrating greater efficiency compared to plasmid form in breast cancer cells and leukemia, both in vivo and in vitro.[90] Moreover, Wang et al successfully loaded the RNP form of CRISPR Cas9 into exosomes derived from HEK293T cells.[91]

To enhance the ability of exosomes to deliver large nucleic acids, Lin et al created a hybrid carrier by merging exosomes and liposomes to form a large vector of the gene editing system. To generate sgRNA-loaded exosomes, the researchers first transfected HEK293FT cells with the mRunx2 sgRNA plasmid. Then, the hybrid carrier was formed by incubating the sgRNA-loaded exosomes with liposomes and the dCas9 plasmid with an enhanced green fluorescent protein (EGFP) for 12 hours at 37°C. The generated hybrid exosomes successfully transferred the gene editing system to MSCs while dramatically reducing the targeted gene RNA (mRunx2 gene). This method circumvented the exosome delivery restrictions and increased the efficacy of gene editing in MSCs. These results imply that exosomes might be a potential tool for gene therapy applications when paired with other delivery vehicles.[1] In another study, the exosome's delivery of the gene editing system eliminated the mutant KrasG12D oncogenic allele in pancreatic cancer cells in vitro and in vivo.[92]

It should be noted that step-by-step exosome journey evaluation improves cargo delivery. The engineering of the natural exosomes helps with targeting and clearance avoidance. For example, polyethylene glycol (PEG), a hydrophilic agent, can reduce circulation clearance and increase cell targeting. Despite its positive role, PEG can decrease cell membrane fusion and endosomal escape. However, sheddable PEGylation may improve cell fusion, cargo delivery, and endosomal escape. In experiments using liposomes, pH-sensitive linkers such as diorthoester, vinyl ether, phosphoramidate, hydrazone, and β-thiopropionate caused detachment of PEG in low pH environments such as tumor microenvironments, endosomes, and lysosomes.[93] Along with PEG, overexpression of CD47 on exosomes was shown to reduce macrophage phagocytosis and lengthen the exosome's circulation time. The incorporation of CD47 on the modified exosome demonstrated the considerable cargo impact (siRNA) on KRAS G12D mRNA and decreased pancreatic tumor growth in the mouse model.[14]

Furthermore, exosomes showed the ability to deliver miRNA to target cells. Co-delivery of miRNA-21 inhibitor and 5-fluorouracil through engineered exosomes indicated colorectal anticancer efficiency in mice model.[94] Since exosomal co-delivery of Everolimus and miR-7-5p improved the anticancer effects of Everolimus in lung cancer (non-small cell).[95] In another study, exosomes were decorated with a peptide (KSLSRHDHIHHHC) to target mesenchymal epithelial transition factor (c-MET). Overexpressed c-MET on TNBC intended smart exosomes to deliver doxorubicin with remarkable effectiveness in animal model.[96] Nanotechnology and site-specific targeted drug delivery may overcome tumor cells' immunity, apoptosis, and multidrug resistance.[97] Optimizing cell targeting and intracellular location are crucial components of optimal

medication delivery in addition to biodistribution.[98] Exosomes may become actively targeted carriers due to the surface engineering and cargo loading of exosomes using engineering technologies.

Engineering Exosomes

Exosome engineering involves the manipulation of exosomes to enhance their functionality by loading specific cargo or modifying their surface. Various methods, including biological, chemical, and physical techniques, are employed for creating modified exosomes.[99] Biological techniques involve genetic engineering of the exosome-releasing cells. In this approach, the cells are genetically modified to produce exosomes with desired characteristics. Chemical methods rely on the conjugation of various chemical moieties to the surface of exosomes (Figure 2). This surface modification allows for targeted interactions with specific molecules or cells. Physical techniques, such as sonication, electroporation, extrusion, freeze and thaw, cell membrane permeabilization, and hypotonic dialysis, are utilized to load cargo (Figure 2). Researchers are exploring combinations of these engineering strategies to achieve remarkable results. By combining surface modification techniques with the transport of therapeutic compounds like nucleic acids, proteins, and other particles, enhanced therapeutic potential can be achieved.[100]

Engineering Exosome Through Gene Engineering

Utilizing cell synthesis machinery to express the viral or plasmid vectors can result in surface engineering and loading of therapeutic agents in the exosomes. In this method, the structural proteins and lipids of the exosomes can be exploited as a scaffold to fuse site-specific features. Exosome structural proteins, including CD9, CD63, CD81, and Lamp2, assist in fusing surface modifiers and cargo like miRNA and proteins.[100]

The genetic modification of parent cells might result in the removal or strong expression of CD47 on the surface of exosomes. This modification can have implications for the circulation and lifespan of the engineered exosomes. CD47 plays a crucial role in immune evasion and clearance of exosomes, so altering its expression can affect how long the exosomes remain in circulation.[14] In addition, the hampering topology of exosomal structural molecules can be changed. The sequential splicing of the CD63 gene revealed the crucial function of the transmembrane 3 (TM3) segment in exosome membrane integration. This engineered CD63 can be a suitable platform for introducing the site targeted and cargo portions outside and inside exosomes, respectively.[17] Exosomal membrane proteins can attach to cargo like reporter proteins,[101] DNA vaccination,[101] and cytosine phosphate guanine (CpG) motif.[102]

The surface of exosomes can be engineered by incorporating diverse peptide-based targeting moieties, improving delivery to specific cellular destinations. The fusion peptides can guide exosomes to precise locations within the body. For instance, RVG (rabies viral glycoprotein) allows for brain targeting, RGB for acetylcholine receptor targeting, and M12 for muscle tissues. These fusion strategies can be applied to critical exosomal proteins like CD63, CD81, and Lamp2b, as well as lipid components, ensuring a versatile and practical approach to engineered exosome-based delivery systems.

Exosome Membrane Engineering on Protein Platform

Researchers have used different proteins on the exosome as the platform for engineering their surface. For instance, CD63, CD9, and Lamp2B have been used for exosome engineering.

CD9 as Platform for Exosome Engineering

Yim et al engineered the photo-activated exosomal delivery system. Various proteins, such as the photoreceptor protein CRY2 and its binding protein from the plant Arabidopsis thaliana, were attached to CD9 using a linker of light-controlled protein-protein interaction. The linker induced the binding and releasing of the cargo proteins to the CD9-conjugated exosome in the presence and absence of blue light, respectively. These exosomes efficiently deliver their cargo inside the recipient's cell cytoplasm in vivo and in vitro. In this study, they transfected HEK293T cells with two vectors: one containing luciferase-mCherry-photoreceptor (CRY2: Cytochrome 2) and the other with CD9-EGFP-photoreceptor interacting protein (CRY interacting protein (CIB1)). The blue light activated the cytochrome receptor and its binding protein, allowing the interested protein to be loaded into the exosome. The researchers demonstrated the efficient transport of various proteins, including mCherry, Bax, a super repressor of the IkB protein, and Cre recombinase[13] (Figure 3A).

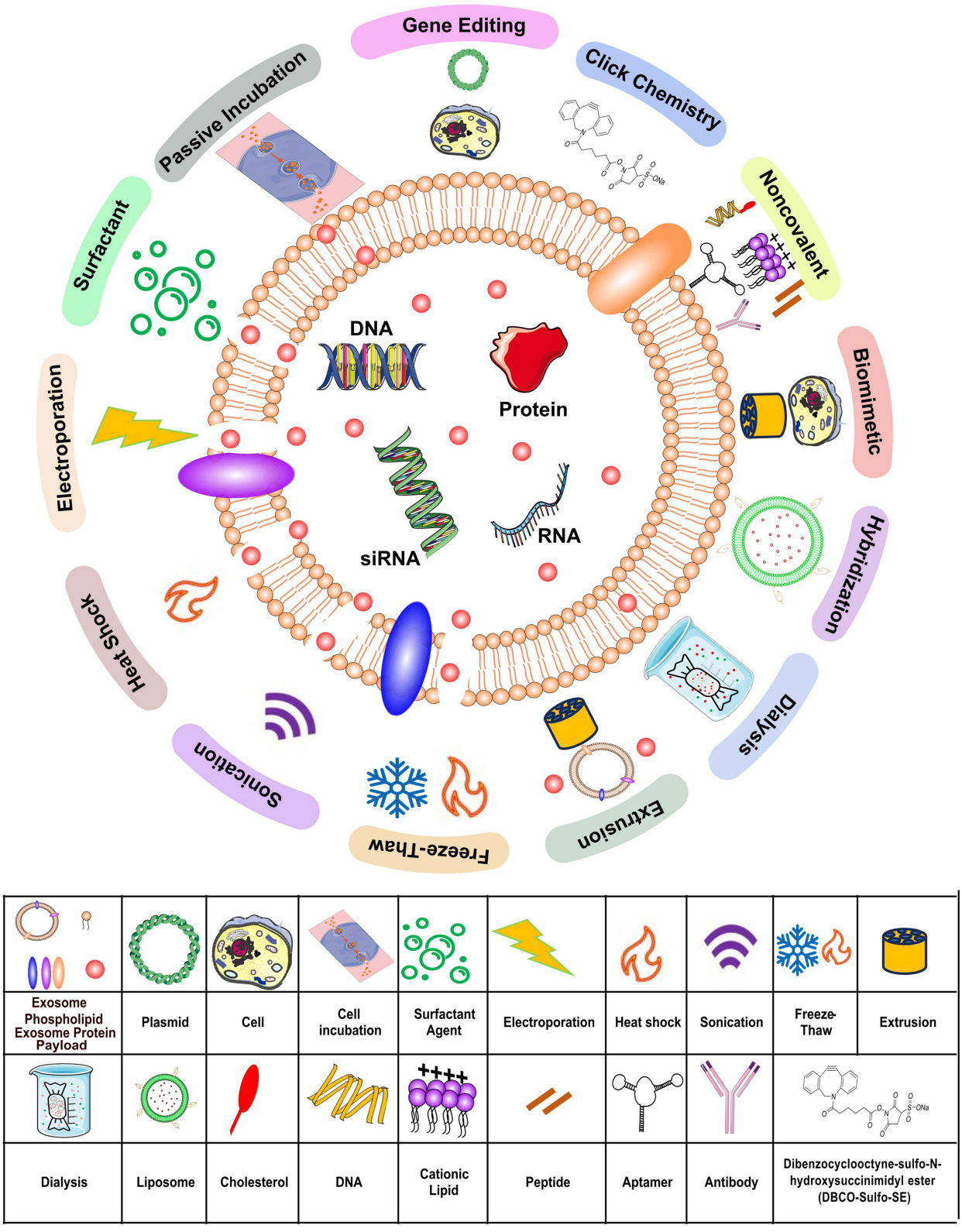

Figure 2 Schematic diagram of exosome engineering for cargo loading and surface modification. Techniques include gene editing, passive incubation, surfactant treatment, electroporation, heat shock, sonication, freeze-thaw, extrusion, and dialysis. Liposome hybridization enables surface and cargo loading. Cell extrusion produces biomimetic exosomes. Surface engineering employs non-covalent agents or click chemistry. One part of the figure was drawn using an image from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution (https://creativecommons.org/licenses/by/4.0/).

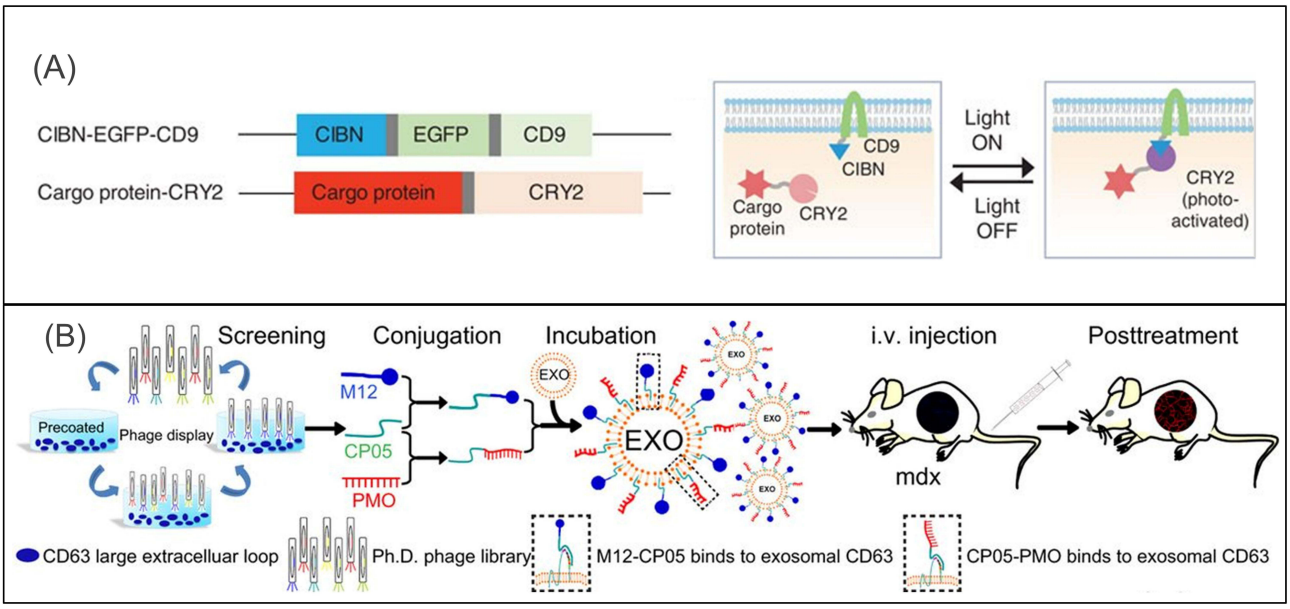

Figure 3 Engineering of the exosome using its protein platform. (**A**) The exosomal CD9 protein was modified with Enhanced Green Fluorescent Protein (EGFP) and Cry Interacting Protein (CIBN). This enables attachment to the photoreceptor protein CRY2 under blue light, and release of the photoreceptor and cargo protein in its absence. Reproduced with permission from Yim N, Ryu SW, Choi K, et al. Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module. Nature Commun. 2016:9.[13] (**B**) Using the bacteriophage library, the peptide CP05 with the best affinity for CD63 was selected. CP05 was conjugated with the muscle-targeting peptide (M12) and phosphorodiamidate morpholino oligomer (PMO). CP05-M12 and CP05-PMO were attached to exosomes, enabling targeting of dystrophy mice models (mdx). "i.v". stands for intravenous. From Gao X, Ran N, Dong X, et al. Anchor peptide captures, targets, and loads exosomes of diverse origins for diagnostics and therapy. Sci Transl Med. 2018;10(444). Reprintedwith permission from AAAS.[103]

CD63 as Platform for Exosome Engineering

Using muscle targeting peptide (M12) on the exosome's surface showed its cargo's significant effect (dystrophin correcting oligomer). In this study, Gao et al found the best exosome-capturing peptide CP05 by the bacteriophage library. CP05, with the sequence of CRHSQMTVTSRL, showed high affinity to CD63 on the surface of the exosome. They conjugated CP05 to muscle targeting peptide (CP05-M12). They also conjugated an exon-skipping oligo (phosphorodiamidate morpholino oligomer (PMO)) to CP05 using amid linker (CP05-PMO). The oligo antisense was used to mask exon 23 of the dystrophin protein. The CP05-M12 and CP05-PMO were incubated with exosomes and attached to CD63 on their surface. Following this engineering, dystrophin expression increased 18-fold in dystrophin-deficient mice[103] (Figure 3B).

In another study, CD63 was attached to the gene-editing system CRISPR Cas9. Li's group fused exosomal CD63 to GFP in one plasmid and gene editing components anti-GFP in another. The affinity of GFP and its antibody facilitated the loading of gene editing cargo in the exosome and efficiently edited the red fluorescent reporter gene. In this study, transfected cells produced exosomes in that CD63 linked to Cas9 protein by the GFP and anti-GFP bridge, respectively.[104]

In a distinct endeavor aiming to overcome challenges in attaching peptides to nucleic acids, Han et al introduced the exosomal anchor aptamer (EAA). EAA efficiently binds to proteins on exosomes from various sources, facilitating the effective loading of different nucleic acid drugs onto them. EAA's strong affinity for exosomes and its capability to load nucleic acid drugs, such as the thrombin DNA aptamer inhibitor NU172 and Duchenne Muscular Dystrophy (DMD) phosphorodiamidate morpholino oligomers (PMOs), led to enhanced serum stability and therapeutic efficacy in dystrophic mice following systemic administration at low doses. However, this approach needs more deep research and optimizations.[105]

Lamp2B as Platform for Exosome Engineering

CD107b or Lamp2B (lysosomal associated membrane protein 2b) is another protein for exosome engineering. The ankyrin repeat protein (DARPin) was fused to Lamp2 and used to target HER2+ breast malignancy.[12] Another approach to Lamp2B-based targeting of breast cancer cells involves fusing the iRGD peptide (CRGDKGPDC) to Lamp2B. This engineering on immature dendritic cells' exosome efficiently targeted αv-integrin in MDA-MB 231 cells and delivered Doxorubicin in vitro and in vivo.[106]

Xing et al also employed Lamp2b to attach a HER2-specific single-chain antibody (P1h3) to exosomes, creating a delivery system for cancer immunotherapy. This system encapsulates GSDMD-N mRNA and incorporates Chlorin e6 (Ce6) and HER2 antibodies. By using puromycin in donor cells, they prevented premature mRNA translation, ensuring the stability of the therapeutic payload until it reached the target HER2-positive breast cancer cells. Upon delivery, sonodynamic therapy activates Ce6, neutralizes puromycin, and initiates mRNA translation. This triggers pyroptosis—an inflammatory form of apoptosis that stimulates a potent immune response, significantly suppressing tumor growth in breast cancer mouse models.[24] Notably, Ce6 can induce reactive oxygen species (ROS), inhibit cell proliferation, and cause apoptosis through photodynamic therapy.[107]

Mentkowski designed cardiosphere-derived cells (CDCs) to secrete exosomes containing Lamp2b coupled to the cardiomyocyte-specific peptide (CMP). However, the highest distribution of these engineered exosomes in the lung, spleen, and liver without heart preference in the mice model indicated the low selectivity and specificity of the designed peptide.[108]

Fusing an RNA-binding protein (HuR) to Lamp2b is an alternative for a multi-targeted RNA interference approach. Li et al used HuR to target miRNA 155 and showed its reduction both in vitro and in vivo. The engineered exosome may drive RNA into the lysosome, deactivate it, and affect gene expression. The intravenous administration of these exosomes significantly reduced miR-155 in the liver and spleen and relieved liver fibrosis in the mice model (CCl4-induced). In this study, they mitigated the RNA content of the engineered exosome in an acidified medium to provide more free space for the interested RNA targeting.[109] In another study, Lamp2b was fused to T7 peptide to target glioblastoma. T7 peptide on this engineered exosome targeted the transferrin receptor-rich-glioblastoma cells in the mice model. Furthermore, it efficiently delivered its cargo, miR21 antisense, which caused miR21 mitigation and reduced tumor size.[110]

Lamp2b fusion to RVG (rabies viral glycoprotein) peptide is neurotrophic and targets neural cells in vitro and in vivo.[2] As RVG-engineered exosomes can also target the acetylcholine receptor, the intramuscular administration of these exosomes is used to deliver miR-29 and relieve muscle atrophy and renal fibrosis in the unilateral ureteral obstruction mice model.[111] In another study, the BACE1 siRNA in this RVG-engineered exosome could knock down the BACE1 gene in the Neuro2A cell line and the brain of wild mice. However, it could not target muscle cells through muscle-specific peptide.[2] The engineering of the exosomes and cargo loading is challenging since the fusion of different peptides on Lamp2b showed various outcomes; despite the success of the RVG exosomes, Alvarez-Erviti et al reported the weak abilities of the muscle targeting exosomes.[2] To address this issue and sustain the surface-engineered peptides from degradation, Hung et al showed that the glycosylation group of targeting peptides plays a protective role against lysosomal degradation and higher delivery efficiency.[112] However, the glycosylation motif on the protein platform in another study did not support endosomal escape,[112] a substantial barrier to exosome therapy. Aside from using the protein platform for surface engineering, the lipid part of the exosome membrane is an anchor for the fusion of targeting agents.

Exosome Membrane Engineering on Lipid Platforms

The surface phospholipid of exosomes provides an additional platform for engineering exosomes. This anchor can be conjugated to biotinylated polyethylene glycol for various streptavidin probes or targeting agents.[15] High concentrations of phosphatidylserine on the exosome membrane and its high affinity for C1C2 domain of the lactadherin has been used to fuse the targeting moieties like EGFR[113] and HER2 antibodies.[114] Kooijmans et al showed the selective affinity of the engineered exosome toward EGFR + cancer cells (A431 cells) in co-cultured cells (Neuro2A).[113] Wang et al also demonstrated the significant result of selective binding of the engineered exosome to HER2+ cells in vitro and in mice models. The engineered HEK293FT cells released exosomes with an enzyme mRNA (HChRr6, an Escherichia coli enzyme). Expression of the enzyme in combination with its substrate (6-chloro-9-nitro-5-oxo-5H-benzo-(a)-phenoxazine (CNOB)) killed the targeted HER2+ cells and retarded the transplanted tumor in mice model.[114]

Several studies showed the role of GPI in boosting the antigens binding to exosomes.[115] For example, stabilizing HER-2 attachment on exosomes through the GPI anchor demonstrated a robust immune response against breast cancer in vaccinated mice.[116] In addition, the GPI fusion to IL-12 and anti-EGFR particles and on exosome boosted in vitro T cell proliferation and binding to EGFR-overexpressed tumor cells, respectively.[115] In general, the genetic engineering of the parental cells may result in homogeneous vesicles with desired payloads.[117] However, despite its effectiveness, the safety of viral and plasmid vectors[99] and chemical transfection agents, as well as the lengthy procedures of this approach, pose

challenges.[100] The numerous challenges include safety concerns, inconsistent transfection effectiveness, potential genetic mutations, and limited targeting precision. Furthermore, this bioengineering process is time-demanding and must be repeated whenever a new molecular target is chosen. Therefore, there is a pressing need for accessible, quicker, more efficient, and expandable techniques to shift these nanovesicles from the lab to clinical settings.

Additionally, researchers have employed "click chemistry" to modify the surfaces of exosomes, using chemical groups like alkyne groups[118] and copper-free azide-alkyne cycloaddition.[16] Click chemistry offers benefits like a quick process, hydrophilic moieties, and more specific targeting with minimal changes in exosome size and properties.[119] This approach allows attaching targeting agents to the exosome surface through chemical modifications involving antibodies, aptamers, and small molecules.

Engineering Exosome Using Covalent Binding (Click Chemistry)

The chemical means comprise covalent and noncovalent adjustments. Targeting specific tissues and cell types may be accomplished by "painting" the surface of exosomal lipids and proteins with various linkers. Exosome functionalization chemistry includes thiol-maleimide, EDC/NHS, azide-alkyne cycloaddition, and amidation. Despite the simplicity and speed of chemical methods, the immuno-compatibility of additional material has limited this application.[99] Finding non-toxic reagents without affecting the function and robustness of the vesicles is pivotal.[99]

The researchers utilized a pH-sensitive linker, azide-dibenzocyclooctyne, to coat macrophage exosomes with antibodies that target CD47 and SIRP. These proteins enable cancer cells to evade the immune system, so targeting them offers considerable potential for cancer therapy. Dibenzocyclooctyne-conjugated antibodies and azido-modified exosomes detached from each other in the tumor microenvironment. The released antibodies effectively suppress CD47 on tumor cells and SIRP on macrophages in vitro and in vivo. As a result, this technique enables active targeting and has great potential for therapeutic benefits.[88] Targeting CD47 can cause anemia and blood-related side effects by prompting the body to destroy red blood cells. Hence, it's important to carefully determine the right timing, dosage, and consider individual health conditions. Additionally, administering erythropoietin alongside the treatment can be beneficial in managing these effects in clinical settings.[120]

In another study, incubating MDA-MB-231 cells with mannose-azide resulted in the release of azido-functionalized exosomes. These exosomes displayed a reaction propensity toward dibenzocyclooctyne-coupled fluorescent dyes. This potential ability enabled them to follow the exosomes in vitro and in vivo, offering possible uses in various medicinal sectors.[121]

Surface functionalizing using ligand as a cell-targeted component can be an easy method of exosome engineering. Sequential incubation without challenging steps of gene engineering can modify the exosome. The peptide of c(RGDyK) (cyclo(Arg-Gly-Asp-D-Tyr-Lys)) with high affinity to specific integrin (alpha v beta 3), targeted tumor cells in albumin-based nano-drug,[122] then Tian et al utilized this peptide for surface functionalizing of exosomes to aim the cerebral endothelial cell. The azide-functionalized c(RGDyK) interacted with dibenzocyclooctyne on the exosomal amino group (proteins or phosphatidylethanolamine). This curcumin-loaded carrier efficiently arrested the apoptosis and inflammation in the mice model of the ischemic brain and demonstrated successful targeting and therapeutic benefits.[16]

Xing et al proposed a strategy to sequester engineered exosomes away from the fast bloodstream elimination. They modified MSCs- derived exosomes by attaching azide groups using metabolic glycan engineering. Additionally, they modified a collagen hydrogel with dibenzocyclooctyne (DBCO) molecules. The azide-labeled exosomes were then anchored to the DBCO-modified collagen using click chemistry. Subcutaneous implantation of the modified complex of exosomes and collagen hydrogel improved angiogenesis following the release of MSC exosome contents.[123] However, further research and development are necessary before clinical application. Concerns about potential immune reactions to azide-exosomes or modified collagen also need to be addressed through thorough animal studies.

Engineered Exosomes Using Noncovalent Methods

Noncovalent methods of exosome engineering include hydrophobic and electrostatic integration, aptamer and peptide anchoring, and receptor and ligand interaction.[124]

Engineered Exosomes Using Hydrophobic Integration

Hydrophobic integration of the cholesterol-conjugated components is another surface modifier for signal amplification of colorimetric sensor[125] and a targeted delivery system.[126] He et al used cholesterol to anchor a DNA probe and improved the detection of exosomes by biotinylated oligonucleotides and streptavidin-labeled horseradish peroxidase (SA-HRP).[125] The cholesterol-conjugated oligonucleotide is a useful anchor-spacer for attaching agents like small dyes, aptamers, and antibodies (Figure 2). DNA is a promising spacer for exosome modification due to its negative charge and capability for base pairing, as well as its configurable size and controlled release qualities. Yerneni et al induced in vitro and in vivo apoptosis in tumor cells through surface engineering of the exosome and conjugating cholesterol-biotinylated oligonucleotide-streptavidin Fas ligand.[126]

Engineered Exosomes Using Electrostatic Interaction

Nakase et al utilized the electrostatic interaction between cationic lipids and the exosomal membrane, along with the amphipathic GALA peptide, to enhance the uptake of exosomal cargo (Figure 2). GALA peptide consists of repeating sequences of glutamic acid-alanine-leucine-alanine. Incorporating the GALA peptide and cationic lipids increased cargo internalization. The GALA peptide transforms a helical structure at acidic pH levels, mimicking a viral protein. This helical conformation plays a crucial role in facilitating the escape of substances from endosomes. The GALA peptide exposes a hydrophobic leucine residue by adopting this helical shape. This exposure enables the fusion of exosomal and endosomal membranes, disrupting endosomal stability and the subsequent release of cargo into the cytosol. The negatively charged glutamic acid residues of the GALA peptide interacted with cationic lipids to accumulate on the exosome membrane and increased cellular uptake.[127] However, studies showed that the harmful effects of cationic lipids following membrane thinning and erosion resulted in cell toxicity. So, the amount of the cationic lipid should be regulated to avoid making holes in the membrane.[128]

Engineered Exosomes Using Antibodies

An alternative technique for targeted drug delivery is antibody decoration for specific antigens. For example, since the overexpression of CD47 in cancer cells and interaction with SIRPα (signal regulatory protein α) helps cancer cells evade the immune system, inhibiting this interaction can control various cancers.[129] The exosome-conjugated to anti-CD47 or anti-SIRP showed tumoricidal effects in vitro and in vivo.[130] Although Xie's group optimized releasing this exosome in acidic pH, intravenous treatment targeted all cells and dyed animals in less than a month.[88] Considering that CD47 has an extreme distribution in the body, masking therapy of this target in cancer cells should be intelligent and site-specific and avoid impacting normal cells.[129] Alternative exosome painting molecules include aptamers, ligands, and small compounds. These alternative molecules have several advantages over antibodies, including increased stability and penetration. Natural carriers can use aptamers as highly promising recognition moieties for efficiently delivering medicines or probes. Aptamer-engineered exosomes are another targeted drug delivery option like sgc8 that detects the protein tyrosine kinase 7 (PTK7) in cancer cells.[26]

Engineered Exosome Using Aptamers

Click chemistry is used to conjugate aptamers and modify exosomes for targeted purposes. The optimal aptamer for the target is selected through SELEX (Systematic Evolution of Ligands by Exponential Enrichment). DNA aptamers can bind noncovalently to macromolecules like proteins, and their aptamer affinity is comparable to monoclonal antibodies.[131] The aptamer modifies exosome uptake and cellular interaction, leading to aptamer-exosome internalization through clathrin-mediated endocytosis.[26] CD133 and angiopep-2 RNA aptamers are conjugated to exosomes to target glioblastoma and the blood-brain barrier. The modified carrier showed a higher distribution in the brain, although it is also distributed in mice's liver, kidney, spleen, lung, and heart.[132] Additionally, combining gold nanorods and aptamer on the exosomes could increase the efficiency of photothermal killing of cancer cells.[133]

Aptamers offer several advantages over antibodies, including greater immune compatibility, sensitivity to specific targets, and deeper penetration. Moreover, aptamers exhibit uniformity compared to antibodies, which can vary between

batches and have a smaller size (10 nm^2 versus 80 nm^2). This smaller size allows for the attachment of multiple aptamers to exosomes.[134] However, there are challenges to overcome for in vivo applications, such as aptamer aggregation, rapid clearance, and short in vivo lifetime due to the protein corona profile of the immune system. Innovative engineering approaches are necessary to address these issues.[135]

Engineered Exosomes Using Small Molecules

Because many cancer cells overexpress vitamin receptors, the receptor's ligands, such as folic acid, can be helpful for exosome engineering.[136] Folic acid may target tumor cells and prevent tumor development. Furthermore, it inhibits tumor metastasis through the folic acid receptor/cSrc-signaling pathway. Feng et al developed a human hyaluronidase-delivering exosome (PH20) that generates low molecular weight hyaluronic acid, attracting the immune system to combat cancer cells. However, this enzyme may also enhance metastatic movement, which can be countered by incorporating folic acid into their system to inhibit its metastasis impact.[137] The alternative selective method for the specific target is glycan. Carbohydrate components of the cell membrane play the recognition role and can be helpful for targeting. Adding sialic acid to the exosome of the mesenchymal stem cells showed their efficient uptake by Hela cells. This is because sialic acid and immunoglobulin (Ig)-like lectins exhibit a strong binding affinity, facilitating the internalization process.[138]

Engineered Exosomes and Cargo Loading

Cargo loading into exosomes can be achieved through passive and active techniques. The passive method involves incubating the desired payload with either the exosomes or the cells that release them. Although this method is straightforward, it could be more efficient. On the other hand, active loading of exosomes involves increasing the membrane permeability using techniques such as electroporation, sonication, freeze and thaw cycles, dialysis, and extrusion. These methods enable more efficient cargo loading into exosomes (Figure 2).

Passive Exosome Loading

Passive loading includes indirect parental cell and direct exosome incubation with the desired agents. In the indirect method, the desired cargo is loaded into the parental cells, which are then naturally packaged into the exosomes released by these cells. In the direct method, the exosomes are incubated directly with the desired agents, allowing for the loading of cargo into the exosomes. In this method, cell or exosome internalization depends on concentration gradients, allowing desired agents to traverse the cell or exosome membrane through passive mechanisms.[139]

Incubation Desired Payloads with the Parental Cell

The incubation of hepatocellular carcinoma (HCC) cells with various anticancer agents resulted in the natural encapsulation of these agents inside exosomes. These exosomes carried anticancer agents and contained heat shock proteins, which improved the cytotoxicity of natural killer cells.[20] In another study, the incubation of the mesenchymal stem cells with Paclitaxel showed the anticancer effect of the released exosome on pancreatic carcinoma. Pascucci et al revealed that after 24 hours of incubation with Paclitaxel, the exosomes released by mesenchymal stem cells naturally encapsulated the drug and exhibited its anticancer effects on pancreatic carcinoma.[21]

Lee et al (2015) employed a method for loading hydrophobic cargo into exosomes by incorporating liposomes into donor cells. They encapsulated the hydrophobic photodynamic therapy agent, zinc phthalocyanine (ZnPc), inside the liposome. Cancer cells were then treated with ZnPc-loaded liposomes for 30 minutes. After 48 hours, the naturally occurring encapsulation of the cargo inside the exosome was isolated. Additionally, the researchers demonstrated that the liposome facilitated the delivery of hydrophobic cargo to spheroids. These exosomes demonstrated the penetration and anticancer effects of the cargo on HeLa spheroids and CT26 tumors in mouse models following irradiation. Notably, the outer cells of the spheroids efficiently wrapped the cargo within exosomes. This mechanism enabled the cargo to penetrate the tumor microenvironment, including avascular regions.[22]

Incubation Desired Payloads with Exosomes

Exosomes have been shown to load agents like Doxorubicin, Paclitaxel, Rhodamine 123, and curcumin efficiently by co-incubation with the desired agent.[23] In a study, curcumin and JSI-124 (cucurbitacin I), both anti-inflammatory agents, were successfully encapsulated in lymphocyte exosomes (EL-4) through a brief 5-minute incubation at 22°C. When administered intranasally, these loaded exosomes displayed anti-inflammatory effects, presenting potential therapeutic applications for conditions like glioblastoma and autoimmune encephalomyelitis (EAE) disease.[140] The loading efficiency of the exosome, regarding the presence of its natural payloads, is low.[23] However, passive techniques can be employed to load exosomes while maintaining the integrity of their membrane.[119]

Active Exosome Loading

The active loading of exosomes causes pores and membrane disruption to load cargo inside the exosome. This physical technique includes incubation with a membrane permeating agent, electroporation, heat shock, sonication, freeze and thaw, extrusion, and hypotonic dialysis.

Incubation Exosome with Desired Payload and Membrane Permeating Agent

Saponin, an amphipathic glycoside, is commonly used to create pores and enhance membrane permeability.[141] Through selective interaction with cholesterol, saponin can remove it from the membrane, creating holes in the cell membrane.[142]

It has been utilized to encapsulate therapeutic cargo, such as catalase, within exosomes. In one study, catalase was successfully loaded into exosomes by shaking them with 0.2% saponin for 20 minutes. This method demonstrated significant loading efficiency and neuroprotective effects in both in vitro and in vivo models of Parkinson's disease.[5] However, it is essential to note that while saponin is effective in loading exosomes, it can also cause exosome membrane disruption, cytotoxicity, and hemolysis in blood cells, which limits its therapeutic application.[143]

Electroporation

Electroporation is a technique that involves applying electric pulses to create temporary pores in the cell, liposome, or extracellular vesicle membranes, allowing the introduction of molecules such as DNA or RNA. In this method, the electrical pulse in the conductive buffer can reorganize the lipids in the cell membrane, leading to the formation of reversible pores.[144,145] The RVG-engineered exosomes have been successfully loaded with siRNA targeting the GAPDH and BACE1 genes, resulting in their knockdown in vitro and wild mice models.[2] Electroporation has been employed to load Doxorubicin into exosomes for tumor targeting in mice models.[106] The mixture of exosomes and Doxorubicin in an electroporation buffer was kept at 4 °C, followed by loading at 350 V and 150 mF and a subsequent 30-minute incubation at 37°C. The encapsulation efficiency achieved was up to 20%.[106] However, optimizing the buffer, pulse, and temperature parameters for efficient cargo loading through electroporation is essential.[19] Wang et al showed the low efficiency of electroporation in the mRNA loading in the exosome.[114]

Heat Shock

Heat shock is a classical method for bacterial transformation and can be employed to make holes in the membrane to facilitate exosome loading.[146] Heat shock can enhance cell fluidity and induce membrane lipid remodeling, forming pores in the cell membrane.[147] Zhang et al demonstrated that the combination of heat shock treatment and $CaCl_2$ facilitated the efficient loading of miRNA into exosomes by inducing holes in the exosome membrane. Heat shock or $CaCl_2$ alone was not effective in loading miRNA into exosomes. Real-time PCR analysis showed a significant increase in miRNA-15a levels in a macrophage cell line treated with the exosome-miRNA complex. In mice, inhalation of the exosome complex led to elevated miRNA-15a levels in alveolar macrophages. The loading process involved exposing the miRNA-15a and exosome mixture to 42°C for one minute, followed by 5 minutes on ice in 0.1 M $CaCl_2$.[148]

Sonication

Ultrasound can increase cell membrane fluidity through transient cavitation.[149] The exosome membrane can be opened to encapsulate molecules like siRNA using pulsed, high-frequency sounds. Lamichhane et al (2016) successfully delivered siRNA targeting HER-2 using exosomes loaded through sonication. By subjecting exosomes to 35 KHz for 30 seconds, they achieved significant siRNA loading, suppressing HER2 mRNA and protein. Compared to electroporation, sonication was a more efficient method of exosome loading without causing aggregation.[150] In another study, exosomes derived from M1-polarized macrophages were loaded with Paclitaxel using sonication. The loaded exosomes demonstrated a remarkable antitumor effect on cancer cells in vitro and in vivo. Additionally, the loaded exosomes activated macrophage-mediated inflammation, further enhancing the antitumor activity of PTX.[151]

Freeze-Thaw

Freeze-thaw cycles can create transient pores in the cell membrane. This technique, characterized by three cycles of alternating room temperature and −80°C steps, has been used to load agents inside exosomes. However, this method showed low loading efficiency and caused exosome aggregation when encapsulating catalase to treat Parkinson's disease.[5] Tran et al developed a modified freeze-thaw method by incorporating incubation and sonication. They could encapsulate aspirin within exosomes with an 88% encapsulation efficiency. To overcome the low efficiency of exosome loading with hydrophobic drugs, they employed amphiphilic polymers to create an amorphous form of aspirin. The modified freeze-thaw method, combined with incubation and sonication, facilitated the encapsulation of amorphous aspirin inside the exosomes.[152] Furthermore, Tran et al utilized exosomes derived from MDA-MB-231 and HT-29 cells, along with an EpCAM aptamer, to enhance targeting. This approach successfully suppressed breast and colorectal cancer in vivo and in vitro.[153]

Extrusion

Extrusion is the technique involving the passing of the desired cells or exosomes on the carbon filters with progressively smaller filter sizes ranging from 400 to 100 nm. The equipment has a heat blocker to mitigate the increasing temperature while running the force for rupturing the cells or exosomes through almost 31 times back and forth crossing samples over the pores. This method can produce artificial exosomes of uniform size.[154] Additionally, performing this in the presence of loading agents can create vesicles loaded with therapeutic cargo.[155] Haney et al successfully encapsulated catalase inside exosomes using extrusion. They demonstrated high efficiency in encapsulation, release, and protection against protease degradation.[5] Similarly, Jang et al utilized extrusion to create exosome-mimics loaded with doxorubicin. These exosome-mimics containing doxorubicin exhibited significant anticancer effects in vivo and in vitro.[156]

Dialysis

Exosomes can encapsulate cargo by employing a dialysis membrane in a hypotonic solution. The hypotonic solution induces cell swelling, stretching the membrane, and forming temporary pores, enhancing membrane permeability. This process involves mixing exosomes with therapeutic cargo, which creates a gradient that promotes the transfer of the cargo into the exosome, leading to encapsulation. However, it is worth noting that although this technique successfully encapsulated porphyrin, it also resulted in exosome aggregation and decreased cellular uptake.[157]

In summary, various methods have been employed to load cargo into exosomes, each with advantages and limitations. The saponin method has shown high effectiveness in loading catalase into exosomes, promoting cellular uptake. Other techniques, such as room-temperature incubation, freeze-thaw, sonication, and extrusion, have also been explored. However, saponin, extrusion, and sonication showed a higher loading rate, prolonged release, and improved cargo stability.[5] In another study, porphyrin was loaded in the exosome through passive incubation and active techniques like electroporation, saponin, extrusion, and dialysis. The efficiency of cargo loading depended on the hydrophobicity of the cargo, with hydrophilic cargo being loaded more efficiently than hydrophobic cargo. The zeta potential of the exosomes also affected cargo loading efficiency. Exosomes derived from MDA-MB-231 and Epidermal stem cells showed a more

negative zeta potential than human mesenchymal stem cells (hMSC) and HUVEC cells. The more negative zeta potential indicated more significant loading during passive incubation. Saponin and hypotonic dialysis have shown significant improvements in loading hydrophilic porphyrin, while intermediate hydrophobicity porphyrin was loaded more efficiently by saponin and hypotonic dialysis compared to other methods. Electroporation, on the other hand, did not induce aggregation during porphyrin loading. Saponin-loaded exosomes exhibited superior cellular uptake, with no significant effects on size and zeta potential, whereas extrusion and hypotonic dialysis affected these parameters.[157]

Overall, passive incubation maintains size and shape but with low efficiency and unwanted content retention. Extrusion and sonication increase cargo loading but may compromise exosome integrity. Freeze-thaw cycles enhance loading efficiency but lead to larger sizes and aggregation. Surfactants and chemical modifications improve loading but may introduce toxicity. Dialysis offers simplicity but with low efficiency and cell uptake.

Artificial Exosomes

While natural exosomes offer several advantages, including their biocompatibility, biological origin, and inherent functionality, they also present challenges, including low yield, expensive and inefficient isolation methods, unwanted cargo, and complex structure. These limitations have prompted researchers to investigate the development of artificial exosomes as an alternative approach.[158] Two types of artificial exosomes include hybrid exosomes and exosome mimics (Figure 2). Hybrid exosomes are formed by combining exosomes and liposomes. This form of artificial exosomes offers a hybrid structure with the advantages of both liposomes and exosomes. On the other hand, exosome mimics represent a distinct category of artificial exosomes that have emerged as an alternative approach.[158]

Hybrid Exosomes

Exosome-liposome fusion is a promising method for targeted delivery. By incorporating various moieties such as antibodies, peptides, probes, fluorescent tags, pegylated lipids, and therapeutic agents, the targeting ability of hybrid exosomes can be enhanced.[27,99] Different methods like incubation, freeze-thaw, extrusion, and sonication can generate hybrid exosomes.[158] The incubation of exosomes and liposomes can provide enough space for the CRISPR/Cas9 plasmid gene editing system. For this purpose, the Cas9 and gRNA vectors were loaded in liposomes and exosomes, respectively, and then transported to mesenchymal stem cells by the hybrid exosome.

Improving exosome carriers could replace adeno-associated virus (AAV) as an effective CRISPR Cas9 carrier. By addressing safety concerns, exosome-based delivery systems offer a safer and more promising option for gene editing applications.[1] Researchers have explored different strategies to enhance exosome uptake by cells. For instance, Sato et al demonstrated that PEGylation residues, achieved by fusing PEGylated liposomes to exosomes using the freeze-thaw method, can increase cell uptake. However, the timing of this procedure is critical to preserve protein integrity.[27] In a recent study by Ning et al a plasma exosome-liposome hybrid was used for COVID-19 diagnosis. The fusion of liposomes, including Sherlock assay components, and plasma exosomes proved an efficient diagnosis method. The liposome contains reverse transcriptase, recombinase polymerase amplification, CRISPR Cas-12, and guide RNA to identify the COVID-related gene (N-gene). The presence of the virus in the plasma exosome activated the CRISPR complex, which in turn cut the nonspecific quenching probes as a marker of COVID-related RNA.[159] Jhan demonstrated that incorporating various synthetic lipids into isolated exosomes can substantially increase particle numbers, ranging from 6 to 43 times. Additionally, they illustrated a remarkable 14-fold enhancement in cell uptake of these engineered exosomes for delivering siRNA to a lung cancer cell line.[160]

The phospholipids in hybrid exosomes primarily come from synthetic and tissue-derived sources (Table 2). Tissue-derived lipids from eggs or cattle pose instability and pathogen contamination risks. On the other hand, synthetic lipids, either entirely synthetic or derived from glycerol-3-phosphocholine (GPC), sourced from plants or animals, offer stability advantages. However, animal-derived GPC may contain contaminants, prompting the use of soybean lecithin as an alternative. Yet, this substitution can introduce stereochemical impurities into the final product.

Despite being costlier and facing scalability challenges, synthetic phospholipids are often preferred due to the unknown impurities in natural sources.[161]

Table 2 The Lipid Sources for Exosome Engineering

Abbreviation	Full Name	Formula	Source of Preparation	Company
Cer	Ceramide	C34H67NO3	Bovine spinal cord	Sigma Aldrich 22,244
Chol	Cholesterol	$C_{27}H_{46}O$	Egg or Wool grease	Sigma Aldrich 228,111
CTAB	Cetyltrimethylammonium bromide	$C_{19}H_{42}BrN$	Synthetic	Sigma Aldrich H5882
DC-Cholesterol	1,2-dioleoyl-*sn*-glycero-3-{[N-(5-amino-1-carboxypentyl)-iminodiacetic acid]succinyl} (nickel salt)	$C_{32}H_{56}N_2O_2$	Synthetic cholesterol	Sigma Aldrich C2832
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphoCholine	$C_{40}H_{80}NO_8P$	Synthetic	Sigma Aldrich P6354
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine	$C_{41}H_{78}NO_8P$	Synthetic	Sigma Aldrich 76,548
DOPS	1, 2-dioleoyl-sn-glycero-3-phospho- L- serine sodium salt	$C_{42}H_{77}NO_{10}PNa$	Synthetic (organic)	Sigma Aldrich P1060
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	$C_{40}H_{80}NO_8P$	Semisynthetic	Sigma Aldrich P0763
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine	$C_{44}H_{88}NO_8P$	Semisynthetic	Sigma Aldrich P1138
DMPC	1,2-dipalmitoyl-sn-glycero-3-phosphoCholine	C36H72NO8P	Synthetic	Sigma Aldrich P2663
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N–[methoxy(polyethylene glycol)2000]	C138H266N2NaO57P	Synthetic	Ananti Polar Lipids 880,128
DSPE-PEG-MAL	*1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethylene glycol)-2000]*	C139H271N4O57P	Synthetic	Ananti Polar Lipids *880,126*
PC	Phosphatidylcholine	$C_{42}H_{80}NO_8P$	Soybean, egg yolk	Sigma Aldrich # 97281–47-5
SM	Sphingomyelin	$C_{41}H_{83}N_2O_6P$	Bovin spinal cord, chicken egg yolk	Sigma Aldrich # 85187–10-6
DOGS-NTA-Ni	1,2-di-(9Z-octadecenoyl)-sn-glycero -3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid)succinyl] (nickel salt);	$C_{53}H_{93}N_2O_{15}Ni$	Synthetic	*Ananti Polar Lipids* 790,404

Exosome-Mimics

Exosome-mimics can be prepared with a similar size range of exosomes and the same components of its derived cell membrane. These exosome-mimics can effectively deliver drugs to cancer cells like exosomes, as demonstrated in Yang et al's review.[162] They can be made through various procedures such as filtration, chemical induction, sonication, freeze-thaw, and mini-extrusion.[163] The selection of cell sources can also affect the carrier's preferred orientation. For instance, the fusion of natural killer cell membranes with liposomes showed effective tumor cell targeting, payload delivery, and improved blood circulation stability. Pichaimani et al showed the effective in vivo and in vitro delivery of doxorubicin in a breast cancer MCF7 model using the NK-92 cell membrane.[164] The hiding function of the NK-92 membrane also improved the tumor targeting of

PLGA (poly (lactic-co-glycolic acid)) nanoparticles. This biomimetic particle's in vitro and in vivo monitoring revealed better tumor cell targeting, blood circulation stability (9 hours), considerable cell uptake, and non-toxicity.[165]

Researchers have coated nanoparticles with membranes from various cells such as erythrocytes,[166,167] platelets,[168] hybridization of both cells,[169] cancer cells,[168] stem cells,[170] leukocytes,[171] and bacteria.[172] Notably, membrane coating only partly covers most of the nanoparticles, yet this partial coating also helps cell targeting and internalization.[173]

Yang et al employed ultracentrifugation and micro extrusion to filter non-tumor MCF10A supernatant through progressively smaller filters (10, 5, and 1 μm filters) to create a uniform exosome-mimetic. Electroporation was used for loading siRNA into the exosome-mimetic, demonstrating excellent site targeting and reduced spleen and liver accumulation in mouse models of MCF7 tumors.[174] Go et al ruptured cell membranes using an alkaline solution containing sodium carbonate and protease inhibitors to eliminate unwanted nucleic acids and cytoplasmic proteins. The resulting membrane sheets were then used to coat nanoparticles, offering a potential strategy for clinical applications.[175]

Several natural sources for exosomes are being explored for engineering applications at the research level (Figure 4). However, identifying a suitable source for artificial exosomes remains a significant challenge in clinical settings.

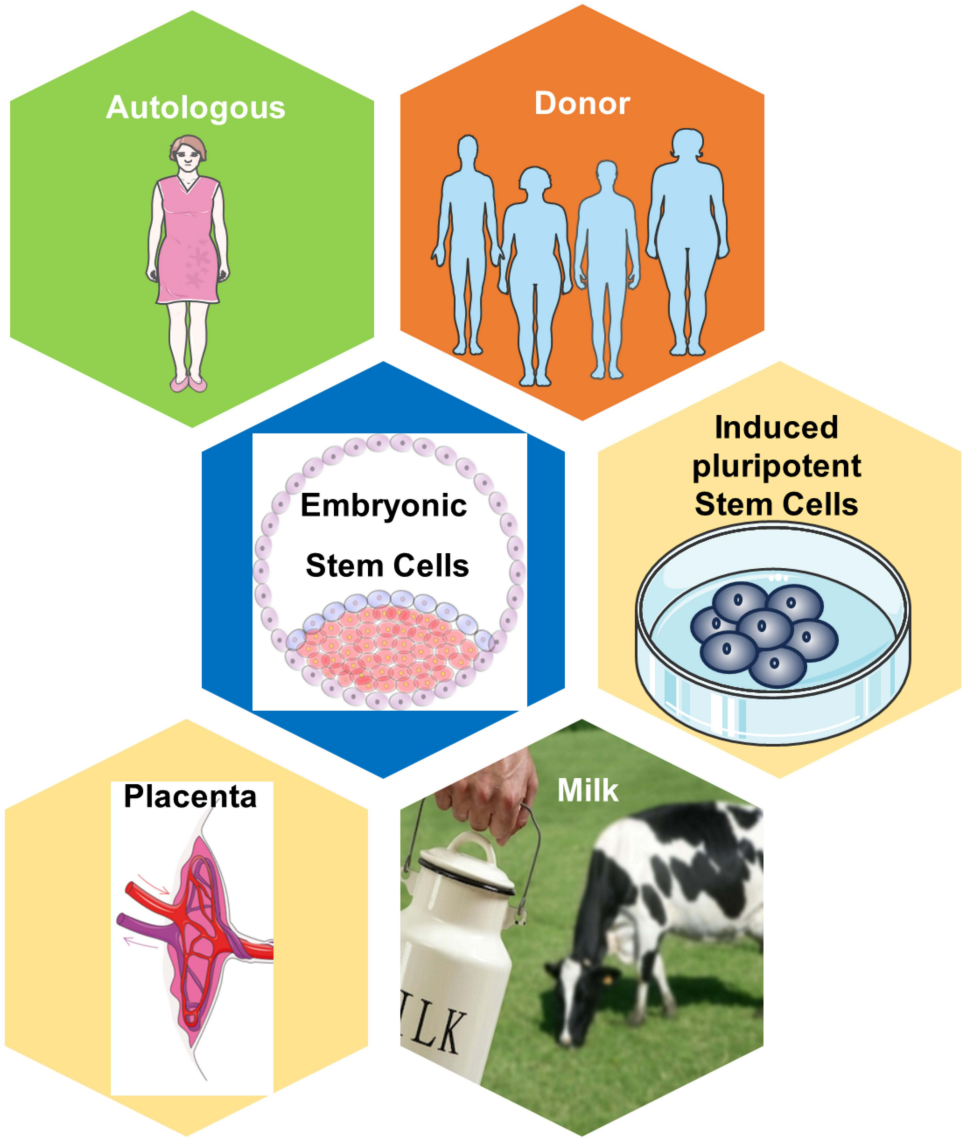

Figure 4 Natural resources for engineering exosomes. These resources include autologous or donor exosomes, embryonic stem cells, induced pluripotent stem cells, placenta, or milk. Parts of the figure were drawn using images from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution (https://creativecommons.org/licenses/by/4.0/).

Munagala et al demonstrated that milk-derived exosomes can be a versatile source for exosomes for drug delivery.[136] In this field, Somiya et al showed that using 1% acetic acid following ultracentrifugation can remove the non-exosome proteins.[176] However, on a large scale, it needs to be optimized. Moreover, there is a risk of pathogen transfer.

Using the autologous exosomes is safer than using the donor cells; however, producing them is time-consuming, cannot be helpful in urgent patient situations, and is on a small scale. Using donor cells provides a larger scale, but there is concern about the potential delivery of the virus, cancer, immune reaction, and contaminations. Different methods have been suggested for the scale-up exosomes, like using disposable or stainless-steel bioreactors for the 2,0001 or 20,0001 scale-up. The closed system, which reduces safety concerns, lacks monitoring compared to the open system.

Placental explants offer another source for large-scale exosome production. These exosomes lack major histocompatibility complex antigens, aiding immune evasion, while they have minor histocompatibility antigens that could promote immune tolerance, thus addressing potential immune incompatibility concerns. However, there is a need for a standardized protocol to evaluate the quality of placental donations and their exosomes. Overcoming these challenges involves implementing standardized screening for pregnancy complications affecting exosome cargo, checking for potential contaminants, and assessing the expected biological activities of isolated placental exosomes.[25]

Embryonic stem cells as an exosome source raise ethical concerns due to embryo destruction. In contrast, induced pluripotent stem cells are less ethically contentious. However, deriving exosomes from in vitro cell culture does not ensure consistent exosome populations. Additionally, the absence of standardized regulations for testing exosome batches leads to each company employing different quality testing assays.[177]

Conclusion and Future Perspectives

Exosomes are gaining attention as natural carriers in diagnostic and therapeutic domains. Because exosomes have the same contents as their parent cells, they are valuable tools for monitoring the physiological and pathological aspects of the body. Exosomes in blood, tears, sweat, milk, and other body fluids may provide noninvasive and early diagnostic options. In addition to their diagnostic function, exosomes can be carriers of different hydrophilic and hydrophobic agents for specific targets. As natural vesicles, exosomes have some advantages over synthetic carriers. First, they are natural carriers and more biocompatible, safer options for drug delivery.[178] Second, like their parent cells, exosomes possess a cell membrane architecture that includes crucial proteins, glycan, and lipids, making them ideal carriers for therapeutic agents. Third, exosomes are highly resistant to endosomal degradation, ensuring their effective delivery to target cells. Fourth, they have the innate ability to cross biological barriers, such as the placental and blood-brain barriers, providing access to difficult-to-reach target sites. Fifth, exosomes offer a promising platform for researchers to engineer and modify their surface and contents. This capability allows for the development of targeted drug delivery systems. Finally, exosomes exhibit natural stability and possess a long circulation life span, making them an attractive option for drug delivery applications. However, this research still needs to be optimized for clinical applications.

Researchers face several challenges, including exosome heterogeneity in size, cargo, function, and donor cells. This heterogeneity needs a standardized isolation technique, which is presently absent. Several isolation methods are available, each with its advantages and disadvantages. Immunoaffinity chromatography provides a greater yield, more specific isolation, and a lower beginning sample; however, it contains protein impurity. Regarding the pros and cons of each method, the selection of the isolation technique is related to the downstream application of the exosome.[179]

Exosome diagnostics need reliable markers and sensitive sensors. The ExoDx Lung (ALK) is the first commercially available exosomal liquid biopsy kit. It can detect non-small cell lung cancer using blood and urine samples. This test examines exosome RNA and circulating tumor DNA at the same time. This kit can substitute invasive tissue biopsy with 80% sensitivity and 100% specificity.[179] Researchers are trying to find a noninvasive method for continuous body monitoring. Recently, researchers at the Terasaki Institute designed a contact lens to capture exosomes in tears.[180] Nonetheless, discovering unique exosome markers for each detection and modification is vital.

Many researchers are interested in the therapeutic potential of exosomes; however, inherent exosome limitations, such as poor targeting, low yields, and limited encapsulation efficiency, have hampered their clinical applications. Modifying and engineering exosomes for a specific target helps address these issues. However, there are various challenges to creating exosomes by gene transfection. Concerns include safety, inconsistency in transfection efficiency, mutagenesis,

and insufficient targeting. Furthermore, custom-designed approaches are crucial due to the unique characteristics of various biological targets, including specific cellular receptors and microenvironments. Consequently, researchers face the demanding task of adapting or creating new design strategies, which can be time and resource-intensive.[181]

Chemical conjugation and hybrid exosomes (exosome-liposome fusion) are alternative engineering techniques; however, they raise concerns about toxicity. In contrast, aptamer-mediated systems have shown promise in the preclinical field. Aptamer decoration of exosomes provides advantages, including small size, uniformity, safety, simplicity of use, cheap, and excellent performance. Despite these advancements, challenges must be addressed before exosomes can be widely used in therapeutic applications. These challenges include the lack of standardized separation and engineering procedures, the absence of unique disease-related markers, the short shelf life of exosomes, and their inherent instability.

Beyond the concern of the exosome sources for artificial use, establishing a standardized and optimal cargo loading method is crucial for maximizing the efficacy of artificial exosomes. The inherent variability in size, composition, and functionality poses a significant challenge to achieving standardization and seamless clinical translation of artificial exosomes.

Furthermore, the purity and yield obtained during isolation procedures can significantly influence the therapeutic potential of artificial exosomes. It is imperative to ensure that artificial exosomes are not only pure but also yield high quantities to meet the demands of therapeutic applications. Equally important is the biocompatibility of artificial exosomes, as any adverse immune responses or toxicities induced in vivo could compromise their safety and efficacy. Hence, addressing these critical factors is essential for realizing the full potential of artificial exosomes in clinical settings.

One significant deficiency in cargo loading is the absence of standardized regulations for evaluating encapsulation and loading efficiencies, which must be addressed in preclinical and clinical settings. Moreover, understanding the intracellular fate of artificial exosomes, including their potential breakdown in endosomes like natural exosomes, is crucial for enhancing their functionality and effectiveness.

In the realm of artificial exosomes, ethical considerations regarding their origin and production methods are crucial. These challenges are further exacerbated by the absence of standardized regulations governing the testing of exosome products, resulting in discrepancies in quality assessment protocols across different companies. Navigating regulatory approval for the clinical use of artificial exosomes poses significant challenges due to their complexity and potential risks. To responsibly use artificial exosomes in healthcare, ethical and societal concerns about their usage should be addressed.

Overall, thorough optimization of artificial exosomes is imperative to navigate the diverse effects on their properties. The absence of a standardized, optimized method in preparation, characterization, and biocompatibility has led to protein impurities, inefficient loading, and potential unknown changes, culminating in various side effects and immune reactions. Realizing the expedited adoption of artificial exosomes in clinical settings hinges on seamless collaboration across diverse fields. This collaborative effort must encompass pharmaceutical design, cellular response dynamics, meticulous toxicological assessments, comprehensive preclinical investigations, and rigorous clinical evaluations. By fostering such interdisciplinary cooperation, we can unlock the full potential of artificial exosomes for the advancement of healthcare.

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Disclosure

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